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Quality assessment of *Tulbaghia* rhizomes

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Abstract

Tulbaghia species are used in traditional medicine in southern Africa. They contain sulphur compounds, which have anti-Candida activity. The sulphur compounds are unstable, so different extraction methods were investigated. Grinding the rhizome material in liquid nitrogen and extraction with ethanol yielded the best results. Eight *Tulbaghia* species were tested and found to contain the same pattern of sulphur compounds on the TLC plate, though in varying concentrations, except *T. simmleri*, for which sulphur compounds could not be detected. This means that more species can potentially be utilised for the drug Tulbaghiae rhizoma. A simple quantitative TLC dilution method was developed, which can be used to ascertain whether the rhizome material contains a sufficient level of sulphur compounds. The effect of storage was investigated. The content of sulphur compounds in the rhizomes decreased fast upon storage, half of the main compound was lost four weeks after harvest. Possible adulterants for Tulbaghiae rhizoma are *Allium sativum* and *Agapanthus campanulatus*. It was not possible to detect adulteration with *A. sativum*, but a simple TLC test could detect adulteration with 10 % *A. campanulatus* material.

Keywords: *Tulbaghia*; Quality; Storage; Adulteration; Antifungal

1. Introduction

The genus *Tulbaghia* L. (Amaryllidaceae, Allioideae) (Chase et al., 2009) includes approximately 22 (Vosa, 2009) to 24 (Archer, 2003) species of mostly rhizomatous plants endemic to southern Africa. The majority of the species are found in South Africa, especially in the Eastern Cape. A distinctive feature of the genus is a prominent corona, a 'crown-shaped' outgrowth or appendage of the perianth. Like other members of the Allioideae, a distinctive 'garlic-like odour' is produced when the leaves or rhizomes are damaged, resulting in the release of cysteine-derived sulphur compounds.

Two *Tulbaghia* species predominantly are used in traditional medicine in southern Africa. Uses include the treatment of fits (*T. alliacea*) (Hutchings et al., 1996), fevers, rheumatism and paralysis (*T. alliacea, T. violacea*) (Watt and Breyer-Brandwijk, 1962), stomach ailments (*T. violacea*) (Hulme, 1954; Batten and Bokelmann, 1966; Burton, 1990), constipation/purgative (*T. violacea*) (Watt and Breyer-Brandwijk, 1962), pulmonary tuberculosis and use as anthelmintics (*T. alliacea, T. violaceae*) (Watt and Breyer-Brandwijk, 1962). With the advent of the HIV-AIDS epidemic, *Tulbaghia* species have also found use for the treatment of oral fungal infections. Studies by Motsei et al. (2003), showed that *T. violacea* had anti-*Candida* activity on three *C. albicans* strains. A study comparing the anti-*Candida* activity of *T. alliacea* and *T. violacea* found *T. alliacea* to be more potent; extracts from both species had fungicidal effects, and it was demonstrated that the active compound was the sulphur-containing compound marasmicin (Thamburan et al., 2006). Three sulphur compounds have been detected in *Tulbaghia* species (Burton and Kaye, 1992; Kubec et al., 2002). Marasmin is the main compound stored in the plant. Upon damage of the plant tissue, marasmin comes into contact with an enzyme, C–S lyase, which converts it to marasmicin. Marasmicin then undergoes further chemical decomposition

Abbreviations: DW, dried weight; FW, fresh weight; HPTLC, high performance thin-layer chromatography; TLC, thin-layer chromatography; VIS, visual light.

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into different sulphur-containing degradation products (Kubec et al., 2002).

The purpose of this study was to develop methods of quality control of *Tulbaghia* rhizomes for use in medicinal preparations. The aim was to ensure that when encountering a batch of rhizomes of unknown origin and identity, it would be possible to determine the suitability for drug use. The main focus of the paper was to ensure a high level of the antifungal sulphur compound in the plant material (thereby excluding *Tulbaghia* species with lower levels of sulphur compounds), and to prevent adulteration with other plant products with a similar visual appearance. The study did not include quality parameters like microbial attack or contamination with foreign matters, as these are common to all pharmacopoeia monographs.

2. Materials and methods

2.1. Plant material

The plant species tested, their synonyms, origin and voucher numbers are given in Table 1. The voucher specimens are deposited at Herbarium of University of Copenhagen, Denmark, [C].

2.2. Extraction procedures

In order to determine which solvent to use for extraction of sulphur compounds, rhizome material of *T. natalensis* was extracted with either Milli-Q water or ethanol 1:10 v/w for 30 min in an ultrasound bath.

For evaluation of extraction procedures the following methods were used: A) rhizome material was frozen with liquid nitrogen and powdered in a mortar and pestle, then extracted with cold ethanol, B) rhizome material was crushed in a mortar and pestle, then extracted with ethanol, C) rhizome material was crushed in a mortar and pestle, then moistened with water, covered and left for 1 h before extraction with cold ethanol, D) rhizome material was crushed in a mortar and pestle, then moistened with water, covered and left for 24 h before extraction with cold ethanol. For all extractions the ratio of *T. violacea* rhizome material:solvent was 1:6.25 w/v and the extraction was done for 30 min in an ultrasound bath. All extracts were filtered through filter paper and taken to dryness under vacuum and redissolved in ethanol to 25 mg/ml.

Extracts for the quantitative dilution method were prepared from material of the various rhizomes using extraction method A. Four g of rhizome material was extracted with 25 ml of ethanol. The extract was filtered and taken to dryness under vacuum, and redissolved in 3 ml ethanol.

Boiled aqueous extracts were prepared by boiling 1 g fresh *T. violacea* rhizome material with 100 ml Milli-Q water for 10 min. After cooling, the extract was filtered through a paper filter.

For the adulteration experiments, underground material of *Allium sativum* and *Agapanthus campanulatus* was frozen with liquid nitrogen and powdered in a mortar and pestle, then extracted with cold ethanol (method A) for 30 min in a ultrasound bath. All extracts were filtered through filter paper and taken to dryness under vacuum and redissolved to 25 mg/ml in ethanol.

2.3. TLC

For the quantitative dilution method, various volumes (10, 8, 6, 4, 2, 1, 0.5, 0.2, 0.1 µl) of extract were applied; for other experiments 40 µl of extracts (equivalent to 1 mg), or 4 µg marasmin was applied to Merck silica gel 60 F_{254} TLC plates for normal TLC, or Merck silica gel 60 F_{254} HPTLC plates for densitometry. Plates were eluted with toluene:ethyl acetate 10:3. Plates were sprayed with 0.45 % palladium-II-chloride in 0.09 % aqueous hydrochloric acid and viewed in VIS for detection of sulphur compounds (Wagner and Bladt, 1996). The HPTLC plates were scanned in a Camag TLC scanner 3 densitometer at 321 nm.

For the anti-*Candida* assay 40 µl extract prepared as in method A and D in Section 2.2 were applied to TLC plates and eluted. Part of the plate was sprayed with palladium chloride to identify sulphur-containing band, which was scraped off and eluted with ethanol. The individual bands were applied in spots

Table 1
Tulbaghia species, synonyms, origin and voucher specimen numbers for the species studied.

Species	Synonyms	Voucher number	Origin
T. acutiloba Harv.		NR530	Simply Indigenous Nursery, South Africa
T. alliacea L.f.		NR492	Hoyland Plant Centre, United Kingdom
T. cominsii Vosa		NR521	Priory Plants, United Kingdom
T. galpinii Schltr.		NR522	Plant Heritage, National Collection of *Tulbaghia*, United Kingdom
T. montana Vosa		NR496	Hoyland Plant Centre, United Kingdom
T. natalensis Baker		NR509	Green Goblin Nursery, South Africa
T. simmlerii Beauverd	*T. daviesii* Grey *T. fragrans* I.Verd. *T. pulchella* P.E.Barnes, illegitimate name	NR498	Hoyland Plant Centre, United Kingdom
T. violacea Harv.	*Omentaria cepacea* Salisb. *T. cepacea* L.f.	NR525	Plant Heritage, National Collection of *Tulbaghia*, United Kingdom

to the centre of individual 5 x 5 cm TLC plates and then used for anti-*Candida* overlayer assay.

For the adulteration experiment, extracts of *A. campanulatus* and *T. violacea* were mixed in different ratios, and 20 µl applied to a TLC plate. The plate was eluted in ethyl acetate: formic acid: glacial acetic acid:water 100:11:11:26), and sprayed with anisaldehyde-sulphuric acid reagent, heated at 105 °C and viewed in VIS.

2.4. Anti-Candida overlayer assay

Candida albicans IMI349010 was maintained on a slant culture of 3.2 % Sabouraud agar. 4 ml sterile 0.9 % NaCl was added to the slant culture, the tube was shaken, and 40 µl of the fungal suspension was added to 10 ml of 3.2 % Sabouraud agar kept molten at 45 °C. The agar was poured over the TLC-plates placed in sterile Petri dishes, and incubated at 33 °C overnight. The plates were then sprayed with 2 mg/ml *p*-iodonitro-tetrazolium-violet (Sigma-Alrich), incubated for a further 6 h and viewed for development of red colour. The experiment was done in duplicate. Amphotericin was used as positive control.

2.5. Storage experiment

Rhizomes were harvested, the aerial parts removed and the rhizomes were left at room temperature for four weeks, one week or processed immediately. Mass at harvest and after storage was recorded. Three *T. violacea* rhizomes were used per experiment. The rhizomes were frozen in liquid nitrogen, extracted with ethanol, and extracts analysed by HPTLC-densitometry.

3. Results and discussion

3.1. Visual appearance of species

There have been several descriptions of *Tulbaghia,* these are reviewed by Vosa (2009). The rhizomes of *Tulbaghia* (Fig. 1) generally appear superficially similar depending on their age, except perhaps those of *T. ludwigiana,* which have woody rhizomes. It is not possible to distinguish the species of *Tulbaghia* based on the visual appearance of the rhizomes at all ages, and therefore a morphological characterisation is not meaningful for securing the identity of the rhizome material.

3.2. Extraction method for sulphur compounds

In order to determine the best extraction method, and to investigate the influence of enzymatic activity on the sulphur compounds during extraction, a set of experiments was set up. Water was not very efficient in extracting sulphur compounds compared to ethanol (results not shown), therefore ethanol was chosen as extraction solvent for further experiments. Fig. 2A shows the TLC plate of the different extraction procedures after spraying with palladium chloride. In this system marasmin, due to the polarity of the compound, remained at the origin. Marasmicin, the enzymatic conversion product, has previously been shown to be the predominant band with an R_f-value of approximately 0.5 (Thamburan et al., 2006). It can be seen that the method where the rhizome material was frozen in liquid nitrogen gave the strongest sulphur bands at R_f 0.54 on the TLC plate (lane A), compared to just crushing the material (lane B). The enzymatic degradation of the compounds took place relatively fast, the band at R_f 0.54 had almost disappeared after 1 h (lane C), and after 24 h (lane D), both bands at R_f 0.45 and 0.54 had disappeared, probably being converted to the band occurring at R_f 1. For all subsequent investigations rhizome material was frozen in liquid nitrogen before extraction.

3.3. Antifungal activity of sulphur compounds

The sulphur compounds in *Tulbaghia* species are not stable, as shown when the crushed plant material was left before extraction. This is due to both enzymatic conversion and chemical degradation of the main sulphur compound, marasmin, found in intact plant material. It was investigated by TLC separation and overlayer assay which of the break-down products have anti-*Candida* activity. Due to strong activity of some bands, making it impossible to ascertain whether all bands in the fungal-free zones were active, it was necessary to isolate the bands and apply them to new TLC plates. The bands at R_f 0.45, 0.54 (marasmicin) and 0.67 were found to be active against *C. albicans* for the extract made immediately after crushing. In the extract made of material that had been subjected to enzymatic reaction, several bands had disappeared and only the band at R_f 0.45 showed activity. Activity was thus associated with the compounds occurring at extraction, whereas the new compounds forming after breakdown were not active.

3.4. Plant species to be included in Tulbaghiae rhizoma based on sulphur compound level

In traditional medicine, two species of *Tulbaghia* are used. It is not uncommon for a drug in a pharmacopoeia monograph to be based on more than one species. The European Pharmacopoeia has several examples of this. The sulphur content of a number of the most common, available *Tulbaghia* species in southern Africa were investigated to determine which species could be included in the drug Tulbaghiae rhizoma. The species tested all showed the same pattern of sulphur compounds on the TLC plate, but in varying concentrations (Fig. 2B), except *T. simmleri*, which had very low levels of sulphur compounds.

Drug monographs operate with minimum levels of active compounds for complying plant materials. The level of sulphur compounds in Tulbaghiae rhizoma should as a minimum be at the level found in the two species used in traditional medicine, *T. alliacea* and *T. violacea*. In many monographs, the test extract is compared to a standard extract, but the main antifungal compound in Tulbaghia, marasmicin, is not stable as it undergoes chemical degradation, rendering it unsuitable as a standard. A quantification method for Tulbaghiae rhizome without comparison to a standard solution would therefore be preferable. To develop a fast, simple method not requiring instrumentation to

assess the content of active sulphur compounds in the drug, a quantitative dilution method was investigated. Extracts were applied in decreasing amounts to TLC plates and the amount at which the antifungal, main sulphur compound at R_f 0.5 (marasmicin) was no longer visible was determined for each species (examples see Fig. 2C). For three species, *T. alliacea*, *T. violacea* and *T. galpinii*, the sulphur compound was detectable when applying 1 µl of the extract. The quantitative dilution method can then be performed by preparing a test extract, applying 1 µl extract, develop the TLC plate, spray with

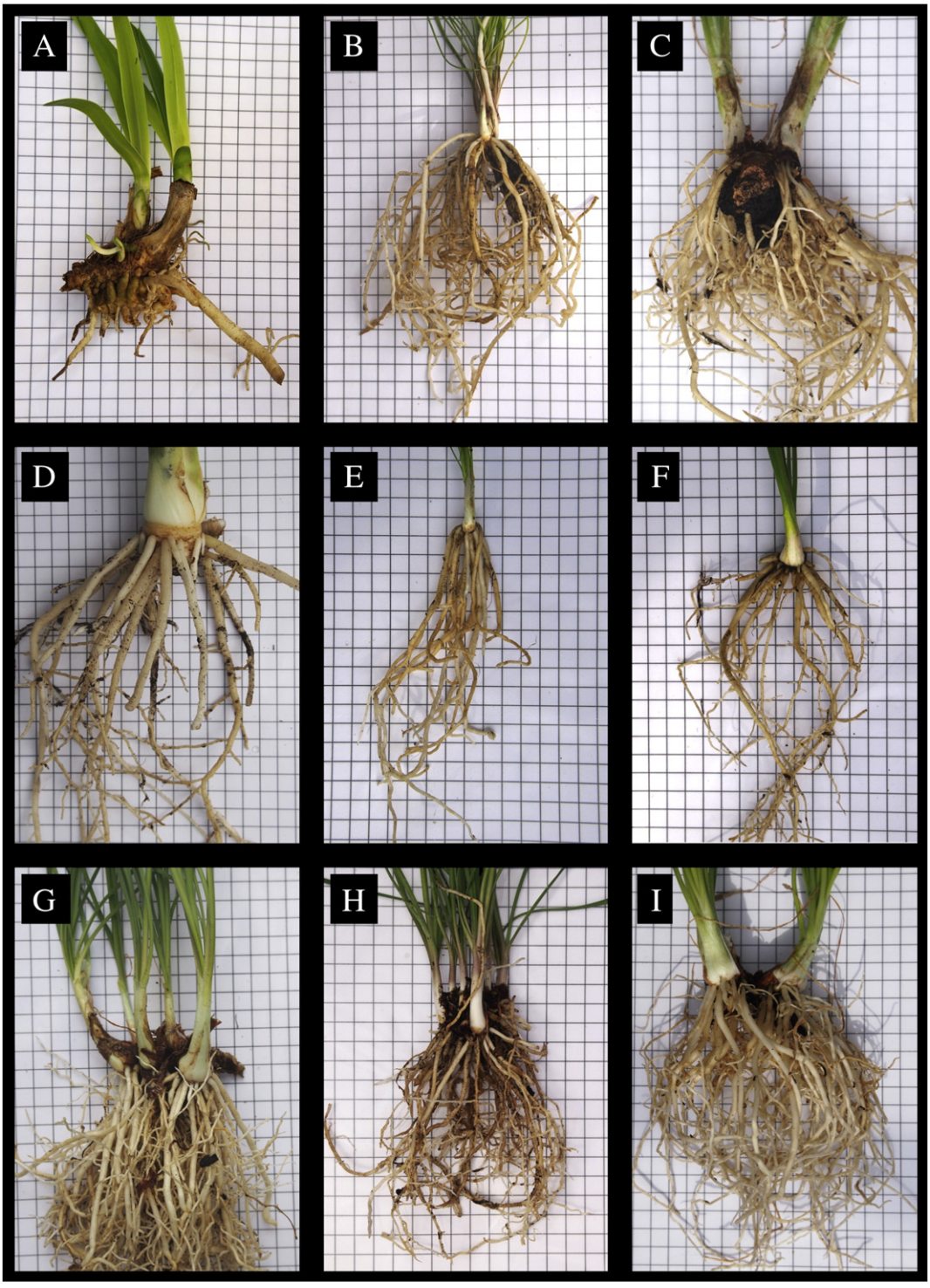

Fig. 1. Underground organs of A) *Agapanthus campanulatus* F.M.Leight., B) *Tulbaghia cominsii* Vosa, (NR521) C) *T. violacea* Harv., (NR525) D) *T. simmleri* P.Beauv. (NR498), E) *T. galpinii* Schltr. (NR522), F) *T. natalensis* Baker (NR509), G) *T. alliacea* L.f., (NR492) H) *T. montana* Vosa (NR496), I) *T. acutiloba* Harv. (NR530) (All photographs by GIS, background grid 10×10 mm).

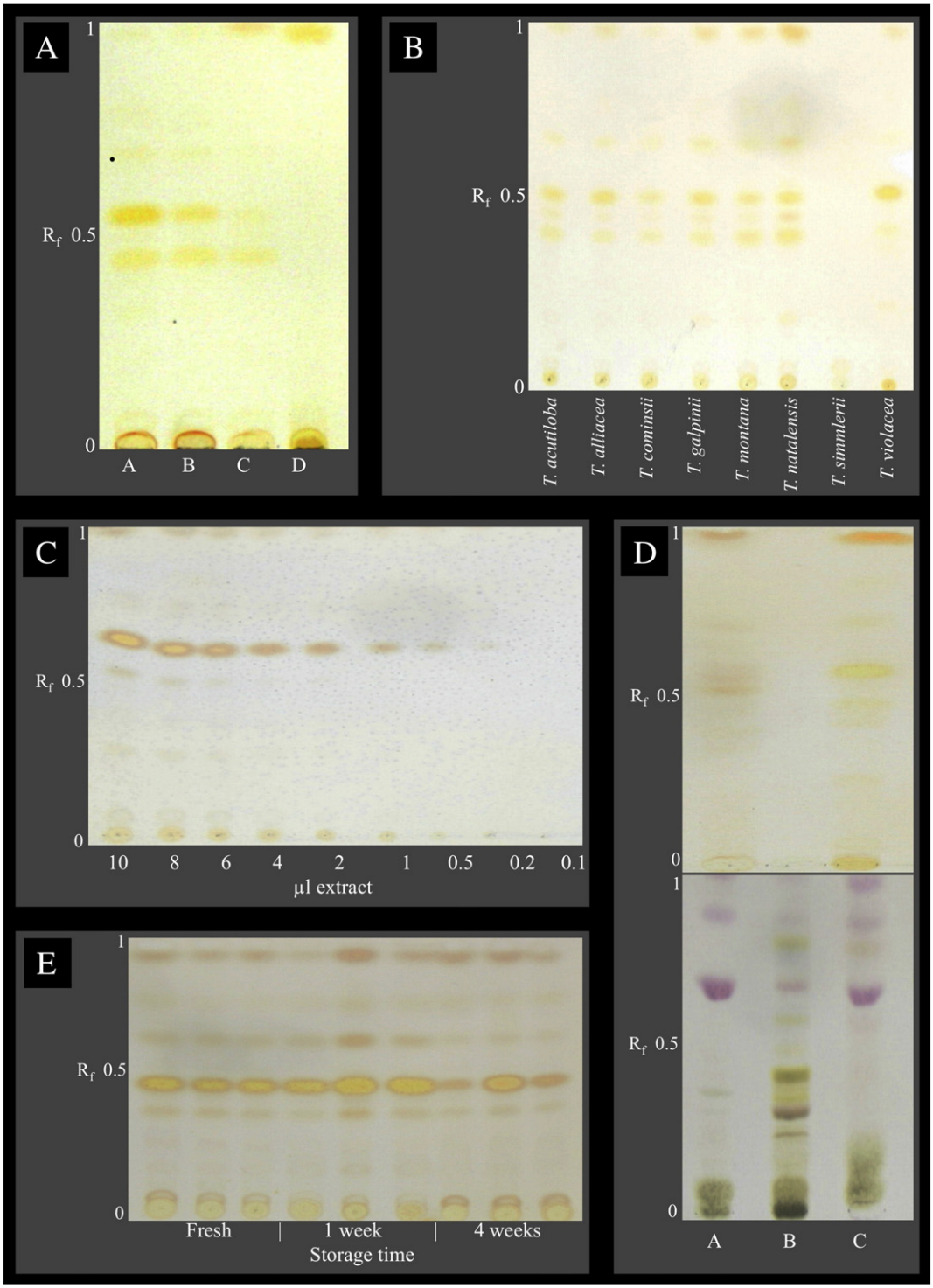

Fig. 2. A: TLC of ethanol extracts of *Tulbaghia violacea* sprayed with palladium-II-chloride : A) bulb material was frozen with liquid nitrogen and powdered, B) bulb material was crushed in a mortar and pestle, C) bulb material was crushed in a mortar and pestle, then moistened with water, covered and left for 1 h before extraction, D) bulb material was crushed in a mortar and pestle, then moistened with water, covered and left for 24 h before extraction. B: TLC of ethanol extracts of *Tulbaghia* species sprayed with palladium-II-chloride showing varying concentrations of sulphur compounds. C: Quantitative dilution of extract of *T. violacea* showing sulphur compounds. D: TLC plates of extracts of A) *Allium sativum*, B) *Agapanthus campanulatus* and C) *Tulbaghia violacea.* The upper plate was sprayed with palladium-II-chloride for detection of sulphur compounds. The lower plate was sprayed with anisaldehyde-sulphuric acid reagent. E: Sulphur compounds in extracts of *T. violacea* prepared from bulb material which was fresh (lanes 1–3 from left), stored one week (lanes 4–6 from left) and four weeks (lanes 7–9 from left).

palladium chloride and see if an orange-brown spot is detectable at R_f 0.5, which would then render the plant material acceptable in terms of marasmicin content.

T. alliacea and *T. violacea* are the two species used in traditional medicine, whereas *T. galpinii* has not been recorded as being used. *T. galpinii* is relatively rare and has a restricted

distribution in the Eastern Cape. As *T. galpinii* cannot be eliminated by a test based on the content of marasmicin, future studies ought to investigate the toxicity profile of this species.

3.5. Storage of rhizomes and preparations

From when a rhizome is gathered until it is bought by a patient and prepared for use, several weeks are likely to pass (Mander, 1997; Williams et al., 2000). To investigate whether the quality of the rhizomes deteriorate post harvest, a storage experiment was performed. The areal parts of the plants were removed, as most rhizomes in trade are presented as rhizomes with roots. During the storage, the water loss from the rhizomes was rapid - 90 % of the weight was lost in the first week (Table 2). The TLC analysis of extracts of *T. violacea* rhizomes stored for one and four weeks showed the same pattern of bands as freshly harvested rhizomes (Fig. 2E), but a densitometric scanning of marasmicin, the main sulphur band (R_f 0.54), showed a decrease of 20 % during the first week of storage and the loss of half of the compound after four weeks (Table 2). As this compound might be considered the main compound responsible for the anti-fungal activity, this loss is detrimental to the quality of the drug. The results show that *Tulbaghia* rhizomes should be used as soon as possible after harvest, preferably reaching the patient within a week.

Most medicinal preparations of *Tulbaghia* rhizomes for medicines are heated, either as infusions or decoctions. To treat fits, an infusion is made from *T. alliacea* rhizomes (Hutchings et al., 1996). For stomach ailments either infusions or decoctions are made from *T. violacea* (Hulme, 1954; Watt and Breyer-Brandwijk, 1962; Batten and Bokelmann, 1966; Burton, 1990). *T. alliaceae* is also prepared as a medicated bath for rheumatism, paralysis and reduction of fever (Watt and Breyer-Brandwijk, 1962). *T. acutiloba* is cooked and used to wash incisions (Watt and Breyer-Brandwijk, 1962). Only when used to treat bacterial infections, including *M. tuberculosis*, and for preparing ear-drops are cold water extracts of *T. violacea* used (Watt and Breyer-Brandwijk, 1962; Felhaber, 1997). Instant heating could minimise the enzymatic conversion of marasmin, but prolonged boiling is likely to result in degradation of the sulphur compounds. However, the prevalent traditional preparations of *Tulbaghia* species include heating of the plant material.

To investigate the stability of preparations, boiled aqueous extracts were prepared as described by Motsei et al. (2003) for use in the treatment of oral candidiasis. The concentration of sulphur compounds in the aqueous extracts was below the detection limit for the chemical methods employed, so the degradation could not be followed over time. The studies by Motsei et al. (2003), where the activity of extracts were followed by an anti-fungal assay using three *C. albicans* strains, showed that the extract could be stored for two days. The extract lost activity after three days when kept at 33 °C, but could be stored for four-five days if refrigerated, or kept at lower ambient temperature.

Table 2
Effect of storage on marasmicin content in rhizomes of *Tulbaghia violacea.*

Weeks	Fresh weight (g)	Dry weight (g)	Dry weight (%)	Extration yield (mg/g)	Area of marasmicin peak (AU) *	Marasmicin, relative to start concentration (%)
0	15.20	–	–	6.5	9886	100
1	7.98	0.75	9.4	4.1	7866	80
4	14.51	2.55	17.6	6.6	4981	50

* Area of band at 0.54 (marasmicin) on HPTLC plate measured by densitometric scanning.

3.6. Adulteration

Valuable plant material is at risk of being adulterated with cheaper plant materials. Likely adulterants for Tulbaghiae rhizoma are garlic, *Allium sativum*, and *Agapanthus campanulatus*. It was investigated whether extracts of *A. sativum* and *A. campanulatus* could be distinguished from a *Tulbaghia* extract. *A. sativum* contains sulphur compounds, which by TLC were indistinguishable from those in *Tulbaghia* (Fig. 2D, upper plate), and the plant material's garlic smell is relatively similar to that of sulphur-containing *Tulbaghia* species. TLC plates of *A. sativum* extracts were sprayed with different standard TLC-detection sprays used for secondary metabolites, but no distinguishing band was found which could be used to identify an *A. sativum* extract. This study could not provide a method for detection of adulteration of *Tulbaghia* with garlic. *A. campanulatus* does not contain sulphur compounds (Fig. 2D, upper plate). The ethanol extract had characteristic bands on TLC when treated with anisaldehyde-sulphuric acid reagent seen as a purple and green band at the lower middle zone (R_f 0.2–0.5) of the TLC plate, where no bands were present with pure *Tulbaghia* extract (Fig. 2D, lower plate), which allowed detection of adulteration with 10 % *A. campanulatus* (Fig. 3).

4. Conclusions

Tulbaghia species are used in traditional medicine, probably due to their content of sulphur compounds. In order to avoid decomposition of the sulphur compounds during extraction, the fresh rhizome material should be frozen in liquid nitrogen, before extraction with ethanol. All of the *Tulbaghia* species tested showed the same pattern of sulphur compounds on the TLC plate, though in varying concentrations, except *T. simmleri*, for which sulphur compounds could not be detected. This means that several species potentially could be utilised for the drug Tulbaghiae rhizoma. A simple quantitative TLC dilution method can ascertain whether the material contains a sufficient level of sulphur compounds. The content of sulphur compounds in the rhizomes decreased fast upon storage, half of the main compound was lost four weeks after harvest. Possible adulterants for Tulbaghiae rhizoma are *Allium sativum* and *Agapanthus campanulatus*. It was not possible to detect adulteration with *A. sativum*, but a simple TLC test could detect adulteration with 10 % *A. campanulatus* material.

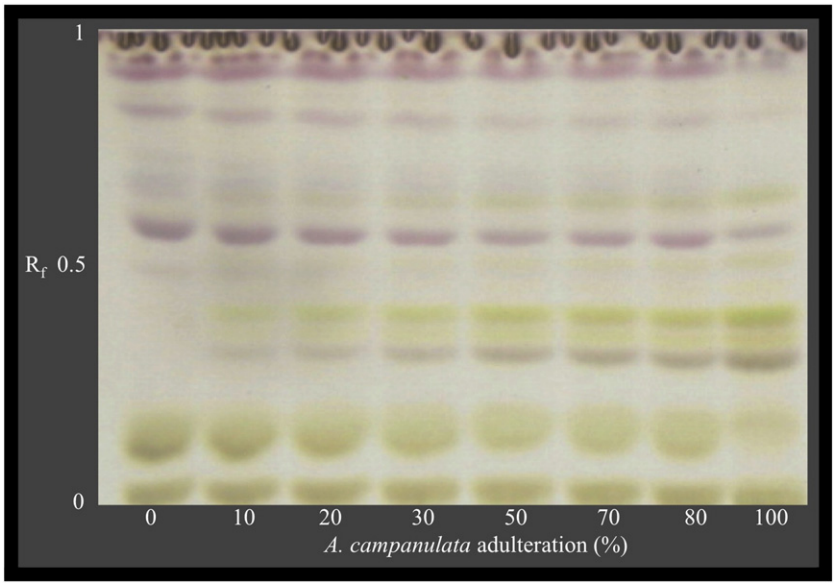

Fig. 3. TLC plate sprayed with anisaldehyde–sulphuric acid reagent showing various ratios of mixed *Tulbaghia violacea* and *Agapanthus campanulatus* extracts.

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